Comparison of the active sites of the purified carnitine acyltransferases from peroxisomes and mitochondria by using a reaction-intermediate analogue

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The carnitine acyltransferases contribute to the modulation of the acyl-CoA/CoA ratio in various cell compartments with consequent effects on many aspects of fatty acid metabolism. The properties of the enzymes are different in each location. The kinetic mechanisms and kinetic parameters for the carnitine acyltransferases purified from peroxisomes (COT) and from the mitochondrial inner membrane (CPT-II) were determined. Product-inhibition studies established that COT follows a rapid-equilibrium random-order mechanism, but CPT-II follows a strictly ordered mechanism in which acyl-CoA or CoA must bind before the carnitine substrate. Hemipalmitoylcarnitinium [(+)-HPC], a prototype tetrahedral intermediate analogue of the acyltransferase reaction, inhibits CPT-II 100-fold better than COT. (+)-HPC behaves as an analogue of palmitoyl-L-carnitine with COT. In contrast, with CPT-II (+)-HPC binds more tightly to the enzyme than do substrates or products, suggesting that it is a good model for the transition state and, unlike palmitoyl-L-carnitine, (+)-HPC can bind to the free enzyme. The data support the concept of three binding domains for the acyltransferases, a CoA site, an acyl site and a carnitine site. The CoA site is similar in COT and CPT-II, but there are distinct differences between the carnitine-binding site which may dictate the kinetic mechanism.

INTRODUCTION

Hemipalmitoylcarnitinium [(2*S*,6*R*)-6-carboxymethyl-2-hydroxy-2-pentadecyl-4,4-dimethylmorpholinium bromide (HPC)] is a conformationally constrained reaction-intermediate analogue inhibitor of the carnitine acyltransferases [1,2] (Figure 1). A reaction-intermediate analogue inhibitor has structural features common to both substrates and products, but should inhibit differently from the reactant and product that it mimics. The racemic form, (±)-HPC, effectively inhibits carnitine palmitoyltransferase II (CPT-II) isolated from rat liver mitochondria with apparent K_i values similar to those obtained for product inhibition by palmitoyl-L-carnitine [1]. (+)-HPC [3], the isomer used in the present work, should be even more effective, because the acyltransferases recognize the chirality at C-3 of the acylcarnitine [4]. As a basis for the comparison of the topographies of the catalytic centres of all of the medium- and long-chain carnitine acyltransferases inhibited by HPC and its analogues, we have studied the kinetics of the inhibition of carnitine octanoyltransferase (COT) and CPT-II.

CPT-II, which is bound to the mitochondrial inner membrane, catalyses the formation in the matrix of acyl-CoA from acyl-L-carnitine imported via the carnitine translocase. Purified enzymes from bovine heart and liver and from rat liver have been characterized [5–8]. Unlike the carnitine acyltransferases acting on cytoplasmic substrates, it is not regulated by malonyl-CoA and not irreversibly inhibited by 2-bromopalmitoyl-CoA in the presence of carnitine [6]. High substrate inhibition, strong product inhibition, the very low K_m values for the acyl-CoA substrates, and complex detergent effects make it difficult to do kinetic studies [9].

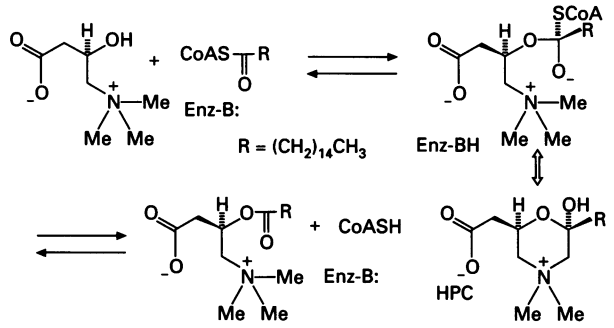

Figure 1 Proposed bio-organic mechanism for acyl transfer in COT and CPT-II

Double-headed arrow indicates structural resemblance between the tetrahedral intermediate and HPC. The OH group on HPC is located where the S in CoA is located in the tetrahedral intermediate. Enz-B: represents a basic group in the active site that serves as an acid–base catalyst.

Despite its popular name, the COT purified from bovine liver peroxisomes is active with all medium- to long-chain acyl substrates [10]. Its role in peroxisomes is not yet clear, but may be connected with removal of chain-shortened acyl groups [11]. COT is distinct from the CPT-II in the mitochondrial inner membrane [6,8] and also from the malonyl-CoA-sensitive CPT-I and microsomal CPT [12]. The purified protein is a monomer of 63 500 Da and does not require detergent for its isolation [13]. However, *in situ*, the enzyme is sensitive to inhibition by malonyl-

Abbreviations used: HPC, (2*S*,6*R*)-6-carboxymethyl-2-hydroxy-2-pentadecyl-4,4,-dimethylmorpholinium bromide (hemipalmitoylcarnitinium); COT, peroxisomal carnitine acyltransferase (octanoyltransferase); CPT-II, the carnitine palmitoyltransferase from mitochondria inner membranes; CMC, critical micelle concentration.

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CoA and may be membrane associated [10]. Studies using substrate analogues suggest that the kinetic mechanism of COT is random order [13]. We have re-examined the mechanism of COT and determined the kinetic mechanism of CPT-II in order to interpret differences between the two enzymes in sensitivity to inhibition by HPC and to begin to understand the structures of the recognition sites in these enzymes.

EXPERIMENTAL

Compounds

Analytically pure (+)-HPC was prepared as described recently [3]. Palmitoyl-L-carnitine, palmitoyl-CoA and deoxycarnitine, from Sigma, St. Louis, MO, U.S.A., were used without further purification. Decanoyl-L-[*N-methyl*-^{14}C]carnitine was synthesized enzymically by a modification of the method of Jalaluddin Bhuiyan and Pande [14]. The reaction was carried out at 37 °C in a shaking water bath and contained (in a final volume of 5 ml): 20 mM potassium phosphate, pH 7.4, 0.5 mg/ml BSA, 0.5 mM 4,4′-dithio(bis)pyridine, 0.25 mM L-[*methyl*-^{14}C]carnitine (53 Ci/mol), 0.45 mM decanoyl-CoA and approx. 5 μg/ml purified COT. The reaction was allowed to go to completion, and any further enzymic reaction was stopped by addition of HCl (0.3 M final concn.). The product was extracted into butanol and freeze-dried. The residue was taken up in water.

Enzyme purification and assays

The enzymes were purified from bovine liver by the published methods for COT [13] and for CPT-II [5].

Forward-direction assay (acyl-L-carnitine formation)

COT and CPT-II activities were determined in the forward direction by a spectrophotometric assay at 324 nm in which the release of CoA was measured by its reaction with 4,4′-dithio-(bis)pyridine as previously described [13]. When examining inhibition by the product, CoA, a direct spectrophotometric assay at 232 nm was used [13].

Reverse-direction assay (acyl-CoA formation)

COT activity was measured in the reverse direction by monitoring CoA ester formation directly at 232 nm [13]. The activity of CPT-II was determined in the reverse direction by measuring the release of L-[^{14}C]carnitine from decanoyl-L-[^{14}C]carnitine (0.05–0.25 Ci/mol). The assay contained, in a final volume of 200 μl in 1.5 ml plastic centrifuge tubes: 20 mM potassium phosphate, pH 7.4, decanoyl-DL-carnitine (50–1000 μM), CoA (50–1000 μM) and approx. 5 ng of purified CPT-II. Triton X-100 was present at a final concentration of 0.005%. After 2 min pre-incubation at 30 °C, the reaction was started by the addition of either substrate. The reaction was stopped by addition of 100 μl of ice-cold 6% $HClO_4$, and 500 μl of cold water-saturated butanol was added. The samples were vortex-mixed for 15 s and then centrifuged for 2 min in an Eppendorf centrifuge. The butanol layer, containing unchanged decanoyl-DL-carnitine, was removed and a portion (250 μl) of the aqueous layer, containing the product, L-[^{14}C]carnitine, was transferred to 10 ml of scintillation fluid for radioactivity counting. The reactions were found to be linear with time and protein concentration for the conditions used. Although decanoyl-DL-carnitine was used in the assay, under the conditions of these experiments the decanoyl-D-carnitine should not affect the measurement of initial rates (see [15]).

Data analysis

For all kinetic studies assays were carried out in duplicate and each experiment was repeated at least once. Kinetic parameters were determined by non-linear regression analysis using the program Enzfitter (Biosoft, Cambridge, U.K.), and the values reported are means from two or more separate experiments. Representative double-reciprocal plots are presented for illustrative purposes.

RESULTS AND DISCUSSION

Kinetic mechanism of COT

The steady-state kinetic mechanism of COT was determined by using initial-rate studies, product inhibition and inhibition by deoxycarnitine, a substrate analogue. The initial-rate data in which the concentration of either substrate was varied in the presence of a series of fixed concentrations of the other resulted in a pattern of double-reciprocal plots that intersect on the abscissa (Figure 2). The pattern of the initial-rate plots is consistent with the random-order rapid-equilibrium mechanism (proposed previously [13]), in which the presence of one substrate does not affect the affinity of the enzyme for the other substrate.

The inhibition patterns and kinetic parameters for COT are summarized in Table 1. The kinetic constants are similar to those previously reported [6,13], but considerably different from those

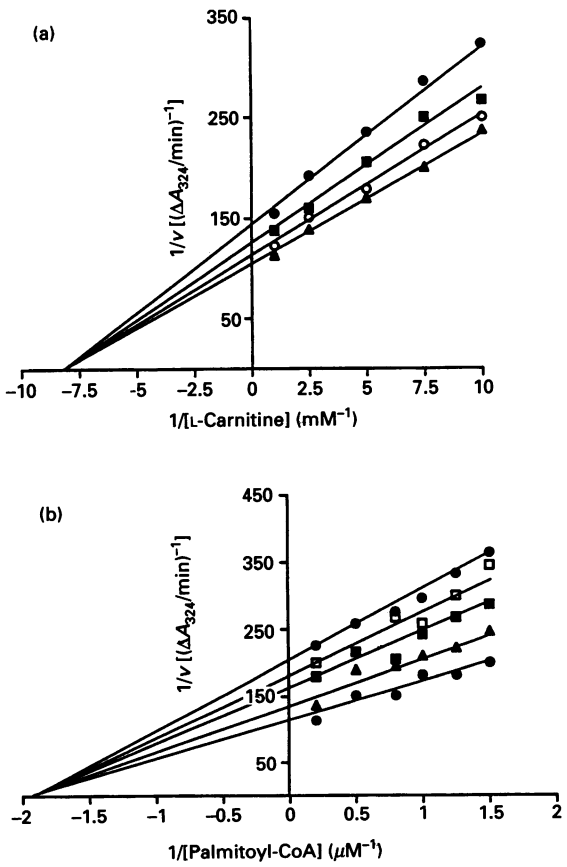

Figure 2 Primary plot for COT

(**a**) The concentration of L-carnitine was varied at a series of fixed concentrations of palmitoyl-CoA: ●, 1 μM; ■, 1.25 μM; ○, 2 μM; ▲, 5 μM. (**b**) The concentration of palmitoyl-CoA was varied at a series of fixed concentrations of L-carnitine: ●, 100 μM; □, 133.3 μM; ■, 200 μM; ▲, 400 μM; ●, 1000 μM.

Table 1 Steady-state kinetic parameters for COT

Abbreviations: Pal, palmitoyl; Carn, carnitine.

	Kinetic constants (means ± S.E.M.) (*n*)		
	K_m PalCoA (μM)	K_m Carn (μM)	$V_{max.}$ (μmol/min per mg)
Forward	0.58 ± 0.02 (10)	108 ± 2 (10)	36 ± 1 (10)
	K_m PalCarn (μM)	K_m CoA (μM)	$V_{max.}$ (μmol/min per mg)
Reverse	7.4 ± 0.65 (3)	16 ± 4 (3)	37 ± 0.25 (3)

Inhibition constants and patterns

Inhibitor	Varied substrate	Fixed substrate	Inhibition pattern	K_i (mean ± S.D.) ($n = 2$)
CoA	PalCoA	Carnitine (saturating)	Competitive	11 ± 4 μM
CoA	Carnitine	PalCoA (saturating)	None	–
CoA	Carnitine	PalCoA (non-saturating)	Non-competitive	16 ± 2.5 μM
Deoxycarnitine	Carnitine	PalCoA (saturating)	Competitive	7.0 ± 1.2 mM
Deoxycarnitine	PalCoA	Carnitine (non-saturating)	Non-competitive	7.2 ± 3.4 mM

obtained in the presence of detergent [15,16]. Although we were unable to carry out a complete analysis of product inhibition by palmitoyl-L-carnitine, the other inhibition patterns observed, in conjunction with the initial-rate data, are consistent with a random-order rapid-equilibrium mechanism and exclude the other sequential mechanisms.

Kinetic mechanism of CPT-II

The kinetic behaviour of CPT-II is notoriously sensitive to the assay conditions used, and it is activated by detergents such as Triton X-100 and Tween-20 [6,17,18]. CPT-II is inhibited by high concentrations of the acyl-CoA substrate when assayed in the forward direction (acyl-L-carnitine formation) and is stimulated by micellar concentrations of acyl-L-carnitine substrate when assayed in the reverse direction (acyl-CoA formation). Although these effects are unlikely to be physiologically significant, they can complicate the interpretation of experimental results, and therefore the choice of assay conditions is critical. We have chosen to study the steady-state kinetics of CPT-II in the reverse direction, using a radiochemical assay. That the K_m values for substrates are higher in the reverse direction facilitates the assay of the enzyme. We observed high substrate inhibition by the acyl-CoA substrate at concentrations as low as 5 μM palmitoyl-CoA. The inhibition by acyl-CoA is manifest as product inhibition in the reverse direction and results in rapid deviation of the time course from linearity, a phenomenon documented by Miyazawa et al. [6]. The use of the more sensitive radiochemical assay avoided the accumulation of inhibitory concentrations of acyl-CoA. Decanoyl-L-carnitine was chosen as substrate because of its favourably high critical micelle concentration (CMC), 5.9 mM [19]. The highest concentration of decanoyl-L-carnitine used was lower than its CMC, thus avoiding the effects of micelle formation. Triton X-100 was present in the assay at a concentration of 0.005% because it is present in the enzyme preparation, but this concentration is well below the CMC of 0.012% [17]. Under our experimental conditions CPT-II displayed simple linear Michaelis–Menten kinetics, and we saw no evidence of the sigmoidal kinetics reported by others [20–22]. It has been shown that sigmoidal kinetics can arise from the use of BSA in the assay medium [23,24], and this may account for the reported sigmoidicity in at least some cases [21,22].

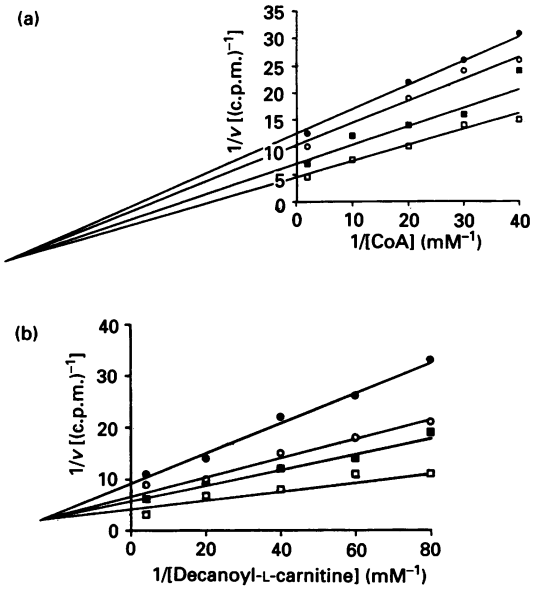

Figure 3 Primary plot for CPT-II, assayed in the reverse direction

(**a**) The concentration of CoA was varied at a series of fixed concentrations of decanoyl-L-carnitine: ●, 33.3 μM; ○, 50 μM; ■, 100 μM; □, 500 μM. (**b**) The concentration of decanoyl-L-carnitine was varied at a series of fixed concentrations of CoA: ●, 33.3 μM; ○, 50 μM; ■, 100 μM; □, 500 μM.

Figure 3 shows the primary plots for the activity of CPT-II

Table 2 Steady-state kinetic parameters for CPT-II

Abbreviations: Dec, decanoyl; Carn, carnitine.

	Kinetic constants (means ± S.E.M.) (*n*)		
	K_m DecCoA (μM)	K_m Carnitine (μM)	$V_{max.}$ (μmol/min per mg)
Forward	3.5 ± 0.2 (4)	1500 ± 100 (4)	14.6 ± 0.4 (4)
	K_m DecCarn (μM)	K_m CoA (μM)	$V_{max.}$ (μmol/min per mg)
Reverse	46 ± 2.8 (8)	112 ± 6 (6)	32 ± 1.2 (6)

Inhibition constants and patterns

Inhibitor	Varied substrate	Fixed substrate	Inhibition pattern	K_i (mean ± S.D.) ($n = 2$)
DecCoA	CoA	DecCarn (saturating)	Competitive	2.5 ± 0.5 μM
DecCoA	CoA	DecCarn (non-saturating)	Competitive	2.0 ± 0.6 μM
DecCoA	DecCarn	CoA (saturating)	None	
DecCoA	DecCarn	CoA (non-saturating)	Mixed	1.5 ± 0.4 μM
Carnitine	CoA	DecCarn (saturating)	Uncompetitive	11.7 ± 1.0 mM
Carnitine	CoA	DecCarn (non-saturating)	Mixed	–
Carnitine	DecCarn	CoA (saturating)	Mixed	11.4 ± 2.4 mM
Carnitine	DecCarn	CoA (non-saturating)	Mixed	–

when decanoyl-L-carnitine and CoA are varied. The intersecting pattern of the primary plots suggests a sequential ordered or random mechanism. A summary of the patterns and K_i values obtained from product-inhibition studies is given in Table 2. Decanoyl-CoA showed competitive inhibition with respect to CoA, suggesting that decanoyl-CoA and CoA bind to the same form of the enzyme. Decanoyl-CoA showed mixed inhibition with respect to decanoyl-L-carnitine when CoA was non-saturating. Inhibition by carnitine was mixed with respect to CoA when decanoyl-L-carnitine was non-saturating, but uncompetitive when it was saturating. The pattern of inhibition shown by carnitine rules out a random-order mechanism or a Theorell–Chance mechanism, since both of these predict competitive inhibition by two substrate/product pairs. Rather, the patterns observed are consistent with a compulsory-order mechanism in which CoA binds first to the enzyme and decanoyl-CoA is the last substrate to leave. This mechanism may be described by the following scheme (Dec, decanoyl; Carn, carnitine):

$$E \leftrightarrow E^{CoA} \leftrightarrow E^{CoA}_{DecCarn} \leftrightarrow E^{DecCoA}_{Carn} \leftrightarrow E^{DecCoA} \leftrightarrow E$$

Although no mechanism has been definitively established for CPT-II until now, characteristics noted in previous work support the ordered mechanism proposed here. Several groups have shown that the concentration of one substrate affects the K_m of the second substrate [7,25,26]. Bremer and Norum [25] have suggested an ordered mechanism as a possible explanation for the high substrate inhibition observed with palmitoyl-CoA. Recently (A. T. Greway, G. P. Heathers, M. E. McDonald, M. Z. Kozak, J. W. Tilley, G. L. Kaplan, R. D. Kierstead, J. G. Millin, R. W. Guthrie, D. F. Kachensky, R. D. Gandour and A. J. Higgins, unpublished work) an ordered mechanism for CPT-I assayed in intact mitochondria has been proposed.

Carnitine exchange into decanoyl-L-carnitine

The exchange of L-[^{3}H]carnitine into the butanol-extractable decanoyl-L-carnitine was measured for several reasons. Firstly, we wished to establish that, as proposed for carnitine acetyltransferase [27], no acyl-enzyme intermediate is formed, i.e., to exclude a Ping-Pong mechanism. The rate constants for the approach to equilibrium in the presence of 10 μg of enzyme protein/ml were 2.8×10^{-3} min^{-1} for COT and 7.6×10^{-6} min^{-1} for CPT-II, both far too slow to be part of the catalytic reaction. With CPT-II some slow acyl-enzyme formation cannot be ruled out, but it is unlikely for COT. The faster rate of exchange observed with COT was not linearly dependent on enzyme concentration and was different for different preparations of the enzyme. The curvature in the dependence of the rate constant on enzyme concentration can be explained by the presence in the enzyme preparation of minuscule amounts of octanoyl-CoA. The last step in purification of COT involves an affinity elution by octanoyl-CoA, which may remain bound even during dialysis. This catalytic amount of acyl-CoA would be sufficient to explain the observed rate of exchange catalysed by COT. For two preparations of COT, the concentration of octanoyl-CoA required to explain the rate of exchange was calculated to be 33% and 22%, respectively, of the enzyme concentration.

A second conclusion can be drawn from the exchange experiment shown in Figure 4. The reaction reached equilibrium at about 2 h, and no further change in the decanoyl-L-carnitine

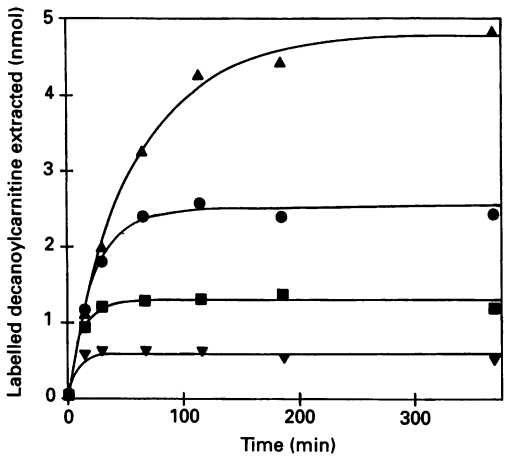

Figure 4 Exchange of [^{3}H]carnitine into decanoylcarnitine catalysed by COT

Reactions contained 1 mM [^{3}H]carnitine (6.43 mCi/mmol), 1.3 mg/ml defatted BSA, 18 μg/ml COT and these concentrations of decanoyl-DL-carnitine: ▼, 12.5 μM; ■, 25 μM; ●, 50 μM; ▲, 100 μM. Reactions were incubated at 30 °C. At intervals, 100 μl portions of the reaction were quenched in 100 μl of 2 M HCl. Amounts of labelled decanoylcarnitine extracted were calculated by using the original specific radioactivity of the L-carnitine. Decanoyl-DL-carnitine was extracted into 250 μl of water-saturated butanol; 170 μl of the butanol layer was transferred to another tube containing 500 μl of butanol-saturated water, and the L-carnitine was back-extracted into the aqueous phase. Then 100 μl of the butanol layer was counted for radioactivity.

level was observed over the next 4 h, nor after 66 h (results not shown). This means that COT does not catalyse hydrolysis of acyl-carnitine. From similar data, CPT-II does not catalyse the hydrolysis, either.

Inhibition of COT and CPT-II by HPC

The inhibition patterns and constants obtained for the inhibition of COT and CPT-II by (+)-HPC are summarized in Table 3. The inhibition was first determined by assaying the enzymes in the reverse direction, because we expected that (+)-HPC, an analogue of the acyl-L-carnitine substrate, would be competitive with respect to this substrate. In the presence of saturating concentrations of CoA, the inhibition was competitive with respect to the acyl-L-carnitine substrate for both COT and CPT-II. The K_i for (+)-HPC was 13 μM with COT (Figure 5a) and 0.16 μM with CPT-II (Figure 6a). When the concentration of decanoyl-L-carnitine was non-saturating, CPT-II showed mixed inhibition by (+)-HPC with respect to CoA (Figure 6b). When assayed in the forward direction, CPT-II showed mixed inhibition by (+)-HPC with respect to both substrates at saturating concentrations of the other substrate (Figure 7). A substrate (or product) analogue such as (+)-HPC would be expected to show uncompetitive inhibition in an ordered mechanism, but these mixed inhibition patterns might be explained by the following reasoning. If we consider the forward reaction:

$$E \leftrightarrow E^{DecCoA} \leftrightarrow E^{DecCoA}_{Carn} \leftrightarrow E^{DecCarn}_{CoA} \leftrightarrow E^{CoA} \leftrightarrow E$$

(+)-HPC, as an analogue of the acyl-L-carnitine product, would be expected to bind to the E^{CoA} complex. The effect of this sequestration of enzyme into a dead-end complex would be to decrease $V_{max.}$ and the K_m of each substrate. Thus the slope of inhibition plots (slope = $K_m/V_{max.}$ at saturating concentrations

Table 3 Inhibition of COT and CPT-II by (+)-HPC

For abbreviations see Tables 1 and 2.

Enzyme	Assay	Varied substrate	Type of inhibition	K_i (mean ± S.D.) (n = 2) (μM)
COT	Reverse	PalCarn	Competitive	13 ± 0.3
	Forward	PalCoA	Competitive	
CPT II	Reverse	DecCarn	Competitive	0.16 ± 0.05
		CoA	Mixed	
	Forward	PalCoA	Mixed	
		Carnitine	Mixed	

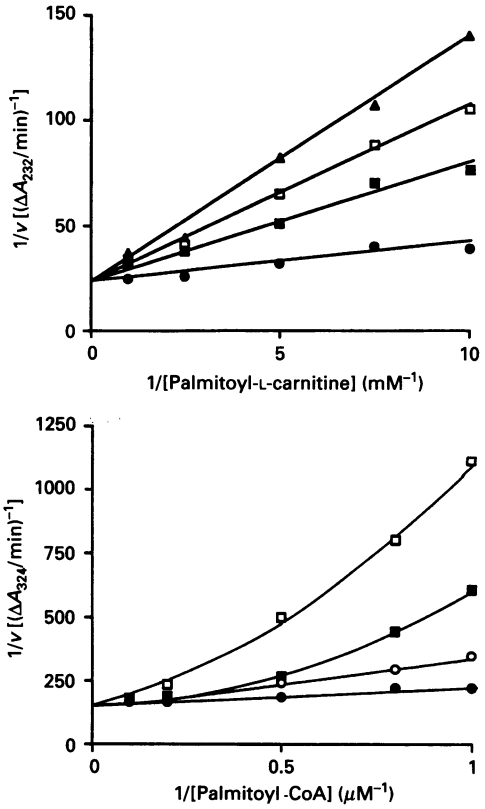

Figure 5 Inhibition of COT by (+)-HPC

(**a**) COT was assayed in the reverse direction. The concentration of palmitoyl-L-carnitine was varied at a series of fixed concentrations of (+)-HPC: ●, 0; ■, 33.3 μM; □, 50 μM; ▲, 66.6 μM. The concentration of CoA throughout was 200 μM (saturating). (**b**) COT was assayed in the forward direction. The concentration of palmitoyl-CoA was varied at a series of fixed concentrations of (+)-HPC: ●, 0; ○, 10 μM; ■, 20 μM; □, 35 μM. The concentration of L-carnitine throughout was 1 mM (saturating).

of the other substrate) would be unchanged, and uncompetitive inhibition would result. However, the mixed pattern observed for both substrates of the forward reaction means that (+)-HPC must bind to the free enzyme and to the $E^{acylCoA}$ complex, thereby increasing the K_m values of both substrates and resulting in mixed inhibition. When assayed in the reverse direction, the mixed inhibition with respect to CoA could arise from (+)-HPC binding to the free enzyme in addition to binding to the E^{CoA} complex.

The inhibition of COT by (+)-HPC in the forward direction

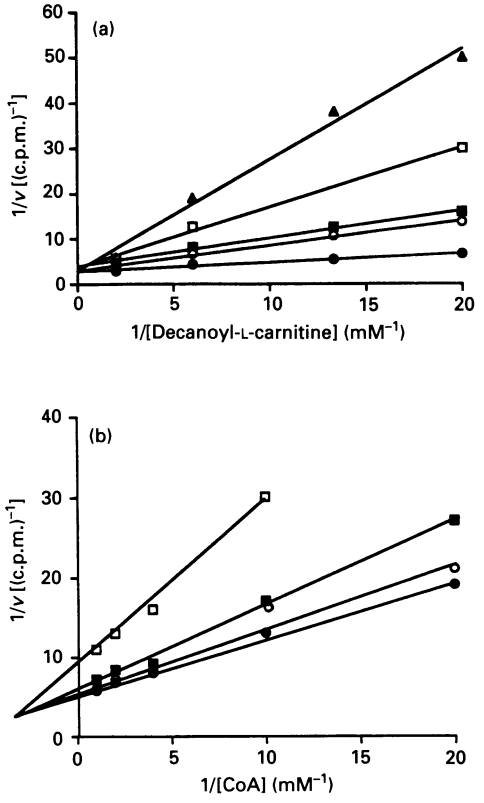

Figure 6 (+)-HPC inhibition of the generation of decanoyl-CoA (reverse reaction) by CPT-II

(**a**) The concentration of decanoyl-L-carnitine was varied at a series of fixed concentrations of (+)-HPC: ●, 0; ○, 0.5 μM; ■, 1 μM; □, 2 μM; ▲, 3.3 μM. The concentration of CoA throughout was 2.5 mM (saturating). (**b**) The concentration of CoA was varied at a series of fixed concentrations of (+)-HPC: ●, 0; ○, 0.125 μM; ■, 0.5 μM; □, 1 μM. The concentration of decanoyl-L-carnitine throughout was 250 μM (non-saturating).

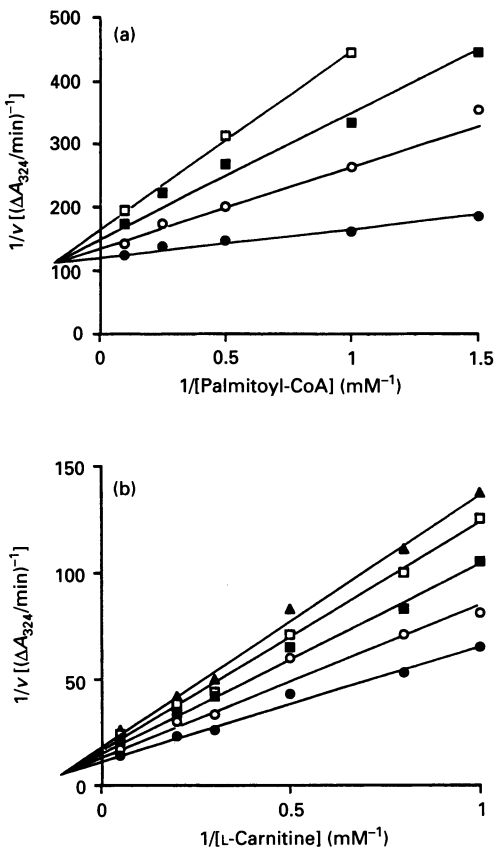

Figure 7 (+)-HPC inhibition of the generation of palmitoyl-L-carnitine (forward reaction) by CPT-II

(**a**) The concentration of palmitoyl-CoA was varied at a series of fixed concentrations of (+)-HPC: ●, 0; ○, 2.5 μM; ■, 5 μM; □, 7.5 μM. The concentration of L-carnitine throughout was 20 mM (saturating). (**b**) The concentration of L-carnitine was varied at a series of fixed concentrations of (+)-HPC: ●, 0; ○, 10 μM; ■, 20 μM; □, 30 μM; ▲, 40 μM. The concentration of palmitoyl-CoA throughout was 37.5 μM (saturating).

was also examined. For COT, much higher concentrations of (+)-HPC were required to effect inhibition, concentrations at which detergent effects interfered with the kinetics, resulting in curvature of double-reciprocal plots (Figure 5b). However, the common ordinate intercept suggests that the inhibition is competitive with palmitoyl-CoA, a pattern that is consistent with a random-order mechanism. It was not possible to determine the type of inhibition with respect to carnitine, because even higher concentrations of (+)-HPC would be required.

The racemic (±)-HPC was previously shown to inhibit CPT-II purified from rat liver competitively with respect to palmitoyl-L-carnitine ($K_i^{app.}$ = 1.6 μM) [1]. Competitive inhibition was also reported versus both L-carnitine ($K_i^{app.}$ = 5.1 μM) and palmitoyl-CoA ($K_i^{app.}$ = 21.5 μM), in contrast with the mixed inhibition reported here. These differences could reflect differences between the enzymes from two sources. However, the authors state that the substrate, inhibitor and detergent concentrations exceeded their respective CMCs, and reported the values as apparent K_i [1].

Comparison of the active sites

By comparing the binding constants for substrates, products and inhibitors of COT and CPT-II some inferences can be made about the similarity of and difference between the active sites of the two enzymes. The catalytic centre contains three distinct binding regions, a CoA site, an acyl site and a carnitine site. The similarity in the values of the binding constants of COT and CPT-II for CoA (K_s = 16 μM and 19 μM respectively) and those obtained for other acyltransferases [6,15,18,28] suggests that the CoA site is similar in all of these enzymes. For COT and CPT-II, the acyl group contributes considerable binding energy, lowering the K_m for the acyl-CoA substrate by about 30-fold compared with CoA and lowering the K_m for the acyl-carnitine by about 15-fold compared with carnitine (see Tables 1 and 2). Carnitine binds to COT (K_m = K_s = 0.1 mM) much more tightly than to CPT-II (from product-inhibition studies, K_i = 11 mM; Table 2). This means that the structure of the carnitine-binding site of COT is well formed in the free enzyme, as might be expected in a random-order mechanism. In contrast, for CPT-II, which follows a compulsory-order mechanism, the carnitine-binding site is less well formed, and indeed carnitine cannot bind unless CoA is bound to the enzyme. Thus CoA binding alters the carnitine site to permit and improve carnitine binding.

For COT, either substrate can bind to the free enzyme, and occupancy of the carnitine site is not affected by occupancy of the CoA site. (+)-HPC can also bind to the free enzyme, but does not bind any better than acyl-L-carnitine. The K_i of COT for (+)-HPC (13 μM) is the same order as the K_m of the corresponding substrate, palmitoyl-L-carnitine (K_m = 7.4 μM). For

CPT-II the K_i for (+)-HPC is 0.16 μM, a value 100-fold lower than that for COT. Although the K_m of CPT-II for palmitoyl-L-carnitine could not be determined due to detergent effects (see above), the K_m for decanoyl-L-carnitine (46 μM) is so much larger than the K_i for (+)-HPC (0.16 μM) that the binding of the inhibitor to CPT-II must be considered an extremely tight and specific interaction compared with substrate binding. This suggests that, at least in the presence of CoA, the carnitine-binding site of CPT-II does not resemble the shape of the substrate or product, but rather that of the proposed tetrahedral intermediate. (+)-HPC, but not acyl-L-carnitine, can bind to the free enzyme in the absence of CoA, which means that its structure permits the interaction (normally induced by CoA) which opens the carnitine site to the ligand. Such complementarity, which is predicted by the transition-state theory of enzyme catalysis [29], attests to the design rationale for (+)-HPC as an analogue of the tetrahedral intermediate of the reaction, but also raises questions as to why (+)-HPC inhibits COT so modestly. Do the atomic positions of the morpholinium ring coincide with those of the tetrahedral intermediate in COT? As mentioned previously about the design of hemiacylcarnitiniums [2], a seven- or eight-membered ring might more closely match the atomic positions of the tetrahedral intermediate. In all of the carnitine acyltransferases that we have studied [1–3], except COT, a hemiacylcarnitinium binds more tightly than the corresponding acyl-L-carnitine. The intriguing observation that the active sites of COT and CPT-II are very different, as revealed by their differential binding of (+)-HPC, prompts further exploration of these enzymes with other active-site-directed inhibitors. The morpholinium framework of (+)-HPC provides an excellent model for the design of inhibitors which will be used to elucidate further the structural basis of the difference between the active sites. Our ultimate goal is to construct a bi-substrate intermediate analogue which will also contain the CoA moiety.

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